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GEOGRAPHIC DISTRIBUTION OF THE MUSCLE-DWELLING NEMATODE *PARELAPHOSTRONGYLUS ODOCOILEI* IN NORTH AMERICA, USING MOLECULAR IDENTIFICATION OF FIRST-STAGE LARVAE

Emily J. Jenkins, Greg D. Appleyard, Eric P. Hoberg*, Benjamin M. Rosenthal*, Susan J. Kutz, Alasdair M. Veitch†, Helen M. Schwantje‡, Brett T. Elkin§, and Lydden Polley

Department of Veterinary Microbiology, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4. *e-mail: emily.jenkins@usask.ca*

ABSTRACT: Molecular identification of dorsal-spined larvae (DSL) from fecal samples indicates that the protostrongylid parasite *Parelaphostrongylus odocoilei* occupies a broader geographic range in western North America than has been previously reported. We analyzed 2,124 fecal samples at 29 locations from thinhorn sheep (*Ovis dalli dalli* and *O. d. stonei*), bighorn sheep (*Ovis canadensis canadensis* and *O. c. californiana*), mountain goats (*Oreamnos americanus*), woodland caribou (*Rangifer tarandus caribou*), mule deer (*Odocoileus hemionus hemionus*), and black-tailed deer (*O. h. columbianus*). The DSL were recovered from populations of thinhorn sheep south, but not north, of the Arctic Circle, and they were not recovered from any of the bighorn sheep populations that we examined. In total, DSL were recovered from 20 locations in the United States and Canada (Alaska, Yukon Territory, Northwest Territories, British Columbia, Alberta, and California). The DSL were identified as *P. odocoilei* by comparing sequences of the second internal transcribed spacer (ITS2) region of ribosomal RNA among 9 protostrongylid species validated by adult comparative morphology. The ITS2 sequences were markedly different between *Parelaphostrongylus* and other protostrongylid genera. Smaller fixed differences served as diagnostic markers for the 3 species of *Parelaphostrongylus*. The ITS2 sequences (n = 60) of *P. odocoilei* were strongly conserved across its broad geographic range from California to Alaska. Polymorphism at 5 nucleotide positions was consistent with multiple copies of the ITS2 within individual specimens of *P. odocoilei*. This work combines extensive fecal surveys, comparative morphology, and molecular diagnostic techniques to describe comprehensively the host associations and geographic distribution of a parasitic helminth.

Protostrongylid nematodes are important parasites associated with recognized disease syndromes and, in some instances, declines in wildlife populations (Lankester, 2001). All protostrongylids have a similar life cycle, in which first-stage larvae are shed in the feces of the mammalian definitive host, invade a gastropod intermediate host, and develop to third-stage larvae that are infective for another definitive host. Species representing 5 genera of protostrongylids (*Elaphostrongylus*, *Parelaphostrongylus*, *Muellerius*, *Umingmakstrongylus*, and *Varestrongylus*) parasitize artiodactyls in North America, and all produce morphologically similar first-stage larvae with a characteristic ''dorsal-spine'' (DSL) (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). The other species of protostrongylids in North America are members of *Protostrongylus* and *Orthostrongylus*, parasitize artiodactyls and lagomorphs, and produce spike-tailed larvae that are clearly distinguishable from DSL but not from each other (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). Length of first- and third-stage larvae has been used to differentiate protostrongylid species; however, measurements overlap among the various species (Kralka and Samuel, 1984; Pybus and Shave, 1984; Gray et al., 1985; Pybus et al., 1989). Consequently, identification of protostrongylid genera or species based on larval length or morphology rarely is possible.

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* U.S. National Parasite Collection and Animal Parasitic Disease Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland, 20705.

† Department of Resources, Wildlife, and Economic Development, Government of the Northwest Territories, Norman Wells, Northwest Territories X0E 0V0, Canada.

‡ Biodiversity Branch, Ministry of Water, Land, and Air Protection, Victoria, British Columbia V8W 9M1, Canada.

§ Department of Resources, Wildlife, and Economic Development, Government of the Northwest Territories, Yellowknife, Northwest Territories X1A 3S8, Canada.

The known geographic distributions of protostrongylid parasites in North America, especially the muscleworms *Parelaphostrongylus odocoilei* and *P. andersoni*, are based on isolated reports and, therefore, often appear to be disjunct (Pybus and Samuel, 1981; Lankester and Fong, 1989; Lankester, 2001). Efforts to define distributions more thoroughly have been constrained by the necessity to kill hosts for recovery and identification of adult male nematodes. Recovery of adult parasites is challenging and tedious, even in heavily infected ungulates, and it often is unsuccessful in lightly infected or atypical hosts (Pybus and Samuel, 1981; English et al., 1985; Lankester, 2001). Presumptive identification based on host and geographic locality is not advisable, both because sympatric hosts commonly share multiple species of protostrongylid parasites and because mixed infections are possible (Carreno and Hoberg, 1999; Hoberg et al., 2002). For example, DSL from white-tailed deer at some locations could represent single or mixed infections of *P. tenuis*, *P. andersoni*, and/or *Varestrongylus alpenae*. Bioassay of unknown first-stage larvae in captive intermediate and definitive hosts, as well as subsequent recovery of adult nematodes, is a reliable method for identification (Pybus et al., 1984; Samuel et al., 1985; Gray and Samuel, 1986) but requires specialized animal care facilities, a supply of captive uninfected hosts, and significant amounts of time (prepatent periods alone can be as long as 3 mo). For these reasons, bioassay rarely is logistically feasible, and antemortem identification of protostrongylids has remained a diagnostic challenge.

Molecular techniques, especially in combination with comparisons of the morphology of adult parasites, are commonly applied to the identification of nematodes (Divina et al., 2000; Blouin, 2002; Monis et al., 2002; Nadler, 2002). The internal transcribed spacers of ribosomal RNA genes (ITS) have been used to differentiate a variety of nematode species in which morphological differences, particularly among larvae, eggs, and adult females, are either subtle or nonexistent (Gasser and Hoste, 1995; Powers et al., 1997; Anderson et al., 1998). Iden-

TABLE I. Specimen type, host, geographic origin, and accession numbers for morphological specimens and sequences of the second internal transcribed spacer region of the ribosomal RNA gene of protostrongylid parasites used as references.

Parasite	Stage	Host*	Geographic origin	Accession numbers
Parelaphostrongylus odocoilei	1 Adult†	DS	Mackenzie Mountains, Northwest Territories, Canada	USNPC 94329 to 94334‡ AY648401 to 648406§
P. odocoilei	4 Adults† and L1‖	SS#	Mackenzie Mountains, Northwest Territories, Canada	USNPC 94891 to 94894 AY648380, AY648392, and AY648393
P. odocoilei	Sequence¶	MD	British Columbia, Canada	AF504031 and AY504037
P. andersoni	L1**	BGC	Beverly herd, Northwest Territories, Canada	USNPC 94890 AY648400
P. andersoni	Sequence¶	BGC	Northwest Territories, Canada	AF504030 and AY504036
P. tenuis	Sequence¶	WTD#	British Columbia, Canada; Minnesota	AF504029 and AY504035
Elaphostrongylus rangiferi	L1††	WC	Gros Morne National Park, Newfoundland, Canada	AY648408
E. rangiferi	Sequence¶	WC	Newfoundland, Canada	AF504027 and AY504033
E. cervi	Sequence¶	RD#	New Zealand	AF504026 and AY504032
E. alces	Sequence¶	MS#	Sweden	AF504034
Umingmakstrongylus pallikuukensis	L1‖	MX#	Kugluktuk, Nunavut, Canada	USNPC 94884 AY648409
Muellerius capillaris	Adult†	DmS	Guelph, Ontario, Canada	USNPC 94888 and USNPC 94889 AY679527 and AY679528§
Varestrongylus alpenae	Adult†	WTD	Riding Mountain National Park, Manitoba, Canada	USNPC 94204 AY648407

* DS, Dall's sheep (*Ovis dalli dalli*); SS, Stone's sheep (*O. d. stonei*); MD, mule deer (*Odocoileus hemionus hemionus*); BGC, barren-ground caribou (*Rangifer tarandus groenlandicus*); WTD, white-tailed deer (*Odocoileus virginianus*); WC, woodland caribou (*Rangifer tarandus caribou*); RD, red deer (*Cervus elaphus*); MS, moose (*Alces alces*); MX, muskoxen (*Ovibos moschatus*); DmS, domestic sheep (*Ovis aries*).
† Identification based on morphology and measurements.
‡ U.S. National Parasite Collection numbers.
§ GenBank accession numbers.
‖ Identification of L1 (first-stage larvae) based on recovery and identification of adult parasites from experimentally infected host.
Experimentally infected host.
¶ Sequence in GenBank from Gajadhar et al. (2000) and Junnila (2002).
** L1 from herd known to be infected with *P. andersoni* (Lankester and Hauta, 1989).
†† L1 from herd known to be infected with *E. rangiferi* (Lankester and Fong, 1989; Carreno and Lankester, 1993).

tification of protostrongylids recently has been attempted using banding patterns in polymerase chain reaction (PCR) of the ITS2 region, and sequences for the ITS2 of 6 protostrongylid species are now available in GenBank (Gajadhar et al., 2000; Junnila, 2002).

Recently, based on comparative morphological studies of adult nematodes, *P. odocoilei* was identified, to our knowledge for the first time, in Dall's sheep, a new genus of host for this parasite (Kutz et al., 2001). This finding in the Mackenzie Mountains, Northwest Territories, Canada, also represented a new geographic record for *P. odocoilei*—approximately 1,000 km farther north than previously reported. This discovery highlighted the need to define more comprehensively the host associations and geographic distribution of *P. odocoilei*. We validated the ITS2 sequence for adult and larval protostrongylid specimens of known identity, and we then obtained and compared ITS2 sequences to identify unknown DSL from feces of wild caprine and cervid hosts across western North America. This combination of morphological and molecular identification led to the first comprehensive description of the geographic distribution of a protostrongylid parasite, and it illustrated the concept of an ''epizootiological probe'' (Hoberg et al., 2001).

MATERIALS AND METHODS

Parasite source and recovery

We obtained reference specimens or previously published sequences representing 7 species of protostrongylid parasites that produce DSL and are present in North America and 2 that could be introduced by animal translocation (Table I). Adult nematodes were identified using comparative morphology and standard criteria for protostrongylids (Boev, 1975; Anderson, 1978; Carreno and Lankester, 1993). Male and female nematodes were cut into sections, and heads and tails were identified and deposited as validated physical vouchers in the U.S. National Parasite Collection (USNPC) at the Animal Parasitic Diseases Laboratory of the Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland. Remaining portions of the body were used for subsequent molecular analyses. First-stage larvae in feces from experimentally or naturally infected hosts from which adult parasites had been identified also were used as representatives of known species. Larvae were recovered from fecal samples using a beaker Baermann technique modified from that described by Forrester and Lankester (1997) by placing a single layer of cheesecloth or single-ply White Swan Quick-Wipes (Scott Paper Limited, Streetsville, Ontario, Canada) between the fecal pellets and a supporting mesh layer. Physical vouchers of larval parasites were deposited in the USNPC. Where possible, molecular data from larvae were validated against that from adult specimens of known identity.

Between 1995 and 2003, collaborators across northwestern North America collected a total of 2,124 fecal pellet groups from thinhorn sheep, bighorn sheep, mountain goats, and cervids (Table II). Samples were collected from the ground or from captured animals and were frozen at −20 C. Both DSL and spike-tailed *Protostrongylus* spp. larvae were recovered using the modified beaker Baermann technique and counted in 3 aliquots of 0.05 ml of the Baermann sediment on a slide under a compound microscope. If very few or no larvae were detected using the aliquot technique, the entire sediment was examined in a gridded Petri dish or on a slide, and all larvae were counted. The proportion of samples positive for each type of larvae (prevalence) was calculated for each sampling location. The DSL were handpicked under a dissecting microscope from at least one individual host from each

TABLE II. Fecal samples from wildlife hosts collected from 1995 to 2003 (n = number of samples), prevalence (%) of *Protostrongylus* spp. larvae (Proto) and dorsal-spined larvae (DSL), and number of second internal transcribed spacer sequences from unknown DSL. Numbers 1–29 represent collections in the present study, letters a through f represent previous studies, in which *P. odocoilei* was confirmed by adult parasite identification. Map column corresponds to Figure 1.

Map	Host*	Locality	Coordinates	n	Proto (%)	DSL (%)	No. sequences	No. hosts	Collectors/source
1	DS	Ivvavik National Park, British Mountains, Northwest Territories, Canada	69°12′N, 140°15′W	6	67	0	—	—	J. Carey, I. McDonald
2	DS	Richardson Mountains, Northwest Territories, Canada	67°55′N, 136°00′W	259	81	0	—	—	J. Nagy
3	DS	Baird and Brooks Mountains, Alaska	67–68°N, 150–159°W†	21	100	0	—	—	L. Adams, C. Kleckner
4	DS	Katherine Creek, Mackenzie Mountains, Northwest Territories, Canada	65°01′N, 127°35′W	650	84	96	15	3	A. Veitch, R. Popko, et al.
5	DS	Yukon-Charley R. Preserve, Alaska	65°N, 143°W†	19	95	0	—	—	J. Burch, J. Lawler
6	DS	Sheep Mountain, Mackenzie Mountains, Northwest Territories, Canada	64°52′N, 127°06′W	22	100	100	1	1	R. Popko, K. Hickling
7	DS	Palmer Lake, Mackenzie Mountains, Northwest Territories, Canada	64°28′N, 129°24′W	36	56	92	1	1	A. Veitch, R. Popko
8	DS	Keele River, Mackenzie Mountains, Northwest Territories, Canada	64°19′N, 126°48′W	1	100	100	1	1	R. Popko
9	DS	Central Alaska Range, Alaska	63°56′N, 147°28′W	7	71	86	1	1	S. Arthur
10	DS	Trench Lake, Mackenzie Mountains, Northwest Territories, Canada	62°38′N, 124°29′W	8	75	100	1	1	K. Davidge, E. Jenkins
11	DS	Nahanni Range, Mackenzie Mountains, Northwest Territories, Canada	61°28′N, 123°20′W	31	90	0	—	—	K. Davidge, A. Gunn
12	DS	Rex Creek, Wrangel Mountains, Alaska	61°19′N, 142°31′W	5	NA‡	40§	3	2	E. Hoberg
13	DS	Tlogotscho Plateau, Mackenzie Mountains, Northwest Territories, Canada	61°07′N, 124°32′W	15	53	100	2	2	D. Tate
14	DS	Goat Creek, Chugach Mountains, Alaska	60°60′N, 142°02′W	3	NA	100	4	2	E. Hoberg
15	DS	Kluane National Park, St. Elias Mountains, Yukon Territory, Canada	60°45′N, 139°30′W†	9	100	100	2	1	J. Carey
16	FS	Faro, Anvil Mountains, Yukon Territory, Canada	62°15′N, 133°15′W	43	56	91	1	1	J. Adamczewski, J. Loehr
17	SS	Muskwa–Kechika Rocky Mountains, British Columbia, Canada	58°45′N, 125°10′W	408	75	75	1	1	T. Ennis, H. Schwantje
18	SS	Spatsizi Plateau, Skeena Mountains, British Columbia, Canada	57°41′N, 129°53′W	4	50	75	3	2	L. Gawalko, H. Schwantje
19	SS	Williston Reservoir, Rocky Mountains, British Columbia, Canada	56°05′N, 122°30′W†	55	95	0	—	—	M. Wood
20	RBS	Cardinal River, Rocky Mountains, Alberta, Canada	53°15′N, 117°30′W	10	100	0	—	—	Hinton Veterinary Clinic
21	RBS	Radium, Kootenay Mountains, British Columbia, Canada	49°56′N, 115°35′W	21	81	0	—	—	H. Schwantje
22	CBS	Fraser River, Lillooet Mountains, British Columbia, Canada	51°19′N, 122°08′W	412	93	0‖	1	1	P. Dielman, H. Schwantje
23	MG	Ramhead, Mackenzie Mountains, Northwest Territories, Canada	62°18′N, 128°58′W	22	50	41	1	1	A. Veitch, R. Popko
24	MG	Tana Lake, Chugach Mountains, Alaska	61°00′N, 142°45′W	3	NA	67	NA	NA	E. Hoberg
25	MG	Ospika River, Rocky Mountains, British Columbia, Canada	56°30′N, 123°55′W	22	55	64	2	1	M. Wood

TABLE II. Continued.

Map	Host*	Locality	Coordinates	n	Proto (%)	DSL (%)	No. sequences	No. hosts	Collectors/source
26	MG	South Coast Mountains, mainland British Columbia, Canada	50°31′N, 124°39′W	18	78	89	2	2	S. Taylor, H. Schwantje
27	WC	Hay River, Northwest Territories, Canada	60°45′N, 116°38′W	7	NA	43	1	1	D. Johnson, B. Elkin
28	MD	Cardinal River, Rocky Mountains, Alberta, Canada	53°15′N, 117°30′W	4	NA	100	5	2	W. Samuel
29	BD	Hopland, California	38°58′N, 123°07′W	3	NA	100	3	3	R. Carreno et al.
a	BD	Coastal Range, California	41°00′N, 123°02′W†	100	NA	25	—	—	Hobmaier and Hobmaier (1934)
b	CMD	W. Sierra Nevada Mountains, California	36°34′N, 118°41′W†	59	NA	98	—	—	Brunetti (1969)
c	MG	Newhalem, Washington	48°N, 121°W	1	100	100	—	—	Pybus et al. (1984)
d	BD	Vancouver Island, British Columbia, Canada	50°N, 126°W	16	NA	56	—	—	Pybus et al. (1984)
e	MD	Jasper National Park and vicinity, Alberta, Canada	52°59′N, 118°06′W	496	NA	93	—	—	Samuel et al. (1985)#
e	MG	Jasper National Park and vicinity, Alberta, Canada	52°59′N, 118°06′W	1	100	100	—	—	Pybus et al. (1984)
e	WC	Jasper National Park and vicinity, Alberta, Canada	52°59′N, 118°06′W	155	NA	28	—	—	Gray and Samuel (1986)
f	MD	Penticton, British Columbia, Canada	49°30′N, 119°34′W	NA	NA	82	—	—	Lankester (2001)

* Abbreviations as in Table I. Also, FS, Fannin sheep (*Ovis dalli stonei*); RBS, Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*); CBS, California bighorn sheep (*Ovis canadensis californiana*); MG, Mountain goat (*Oreamnos americanus*); BD, Black-tailed deer (*Odocoileus hemionus columbianus*); CMD, California mule deer (*Odocoileus hemionus californicus*).
† Approximate map reference based on place name.
‡ NA, not available.
§ Prevalence questionable; samples analyzed under field conditions.
‖ One sample contained DSL, *Protostrongylus* spp. larvae, and eggs of gastrointestinal parasites typical of bighorn sheep, but this composite pellet group may have included feces from sympatric mule deer infected with *P. odocoilei*.
Original identification by Platt and Samuel (1978a).

location and immediately processed for molecular analyses or frozen in tap water at −75 C in 2-ml polypropylene vials (Wheaton Cryule®, Millville, New Jersey).

Molecular analyses

The DNA was obtained from pieces of adult nematodes, individual larvae, or pooled larvae (n = 6–50) by heating to 90 C for 10 min in 10 μl of water and cooling on ice for 25 min. Extraction buffer (20 μl composed of 0.5 mg/ml of proteinase K, 1× PCR Buffer, and 2.5% 2-mercaptoethanol) was added and the mixture incubated at 65 C for 2 hr, followed by heating to 90 C for 10 min. The samples (2 μl/PCR reaction) were used immediately or stored frozen (−20 C).

A PCR modified from that described by Gajadhar et al. (2000) was performed using the primers NC1 (5′-ACG TCT GGT TCA GGG TTG TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Ellis et al. 1986; Gasser et al., 1993). Each 50-μl PCR reaction contained 34 μl of water, 5 μl of 10× PCR buffer, 4 μl of 25 mmol $MgCl^{2+}$, 0.5 μl of 25 mmol dNTPs, 2 μl (40 pmol) of each primer, 0.25 μl (1 U) of Taq DNA polymerase, and 2 μl of sample DNA overlaid with 1 drop of mineral oil. Amplification conditions consisted of an initial 3-min denaturation at 94 C, followed by 35 cycles of 94 C for 60 sec, 60 C for 60 sec, and 72 C for 60 sec. A final extension phase of 72 C for 10 min was followed by cooling to 4 C. Reagent-only (DNA not added) reactions were used as negative controls to detect potential contamination. Reactions were analyzed by electrophoresis through a 2% agarose gel with ethidium bromide staining. Amplification products ranged from 472 to 590 base pairs (bp) and were visualized with an Alpha-Imager gel documentation camera (Alpha Innotech, San Leandro, California).

The PCR products amplified from individual larvae and adult *P. odocoilei* were sequenced directly. In directly sequenced PCR products, overlapping traces were observed at several nucleotide positions. To identify the bases at these positions, DNA was extracted from a single adult female *P. odocoilei* using DNAeasy columns according to the manufacturer's protocol (Qiagen, Valencia, California) and then used as template for PCR. The PCR products from this specimen of *P. odocoilei*, as well as specimens of other adult protostrongylids and all the pooled larvae, were cloned using the Topo TA cloning kit (Invitrogen, Burlington, Ontario, Canada) and sequenced using M13 forward and reverse primers in both directions. Sequence chromatograms were aligned using Sequencher 4.1 (GeneCodes Corporation, Ann Arbor, Michigan) or Seqman and Megalign (DNA Star, Madison, Wisconsin) software, and overall percentage similarities between sequences were recorded. Gapped-BLAST searches of the GenBank database were performed to access previously published sequences (Altschul et al., 1997). Sequences were examined manually for species-specific differences and polymorphic sites.

RESULTS

Selected sequences were deposited in GenBank under accession numbers AY648379 through AY648409, AY679527, and AY679528. Length and raw similarity of ITS2 sequences among the 9 species of protostrongylids are reported in Table III. The 3 species of *Parelaphostrongylus* could be differentiated at 5 sites ranging from single-base substitutions to a 4-base insert and at 2 larger variable regions (Table IV). Single-nucleotide polymorphisms were observed at alignment positions 93 (A or T), 302 (T or C), 334 (C or A), 341 (G or A), and 391 (G or A) in cloned ITS2 sequences of *P. odocoilei* (n = 26). These corresponded to overlapping traces/ambiguous bases when PCR products were sequenced directly (n = 34). In total, we obtained 60 sequences for ITS2 of *P. odocoilei*, 51 from larvae in feces of wild caprines and cervids, and 9 from specimens of known identity.

Based on ITS2 sequences, we identified DSL of *P. odocoilei* from at least 1 host in thinhorn sheep (Dall's and Stone's sheep) at 13 locations in British Columbia, Northwest Territories, Yukon Territory, and Alaska; in mountain goats at 4 sites in British

TABLE III. Size (base pairs [bp]) of second internal transcribed spacer region of protostrongylid species using the primers NC1 and NC2 and percentage similarity among protostrongylids using sequence pair distances from the Clustal V method (Megalign, DNA Star, Madison, Wisconsin). Note that the nonelaphostrongylines were so diverged as to preclude meaningful alignment.

Species	Size (bp)*	Size (bp)†	Size (bp)‡	Po	Pa	Pt	Er	Ec	Ea	Up	Mc	Va
Parelaphostrongylus odocoilei (Po)	571	561	560§	—	91–97	94–97	58–63	53–63	52–55	34–46	31–41	40
P. andersoni (Pa)	566	545	550		—	97–98	51–62	52–55	50–56	35–49	33–41	38–39
P. tenuis (Pt)	576	554	NA‖			—	60–66	53–54	45–53	47–49	38–43	39–42
Elaphostrongylus rangiferi (Er)	597	585	590				—	95	63	40	42	39
E. cervi (Ec)	597	585	NA					—	65	36	40	38
E. alces (Ea)	581	575	NA						—	41	35	39
Umlngmakstrongylus pallikuukensis (Up)	445	445	472							—	51	36
Muellerius capillaris (Mc)	NA	NA	495								—	30
Varestrongylus alpenae (Va)	NA	NA	556									

* Gajadhar et al. (2000).
† Junnila (2002).
‡ Present study.
§ Range 557–562.
‖ NA, not available.

TABLE IV. Fixed differences distinguishing second internal transcribed spacer sequences of the three species of *Parelaphostrongylus* (n = number of sequences). Sequences were aligned using Seqman and Megalign (DNA Star, Madison, Wisconsin) software.

Alignment position	79	84	201	231	346	351	468
P. odocoilei (n = 60)	A	A	G	GATG	A	GAAAA**G**AAAAAAAAG*	TTATTACTAGGT
P. andersoni (n = 17)	G	G	A	—	A	GAAAAAAAAA	TTATTATTATGT or TTAT__GT
P. tenuis (n = 3)	G	A	A	—	C	GAAAAAAAAAAC	TTATTATTATGT

* Most common variant. In a minority of sequences, second G (bold) was substituted by an A, and the number of As was variable (range 8–14).

Columbia, Northwest Territories, and Alaska; and in woodland caribou in Northwest Territories (Table II; Fig. 1). We found no evidence of other species of *Parelaphostrongylus* in wild sheep or mountain goats, but our techniques would not necessarily detect mixed infections, especially at low levels of larval shedding. Prevalence of DSL and *Protostrongylus* spp. larvae in feces of wild ungulates are reported in Table II.

DISCUSSION

Extent of distribution

Parelaphostrongylus odocoilei occupies a far greater geographic range in northwestern North America than has been previously reported. Before its discovery in Dall's sheep of the Mackenzie Mountains (Kutz et al., 2001), the presence of *P. odocoilei* had been confirmed by identification of adult parasites at only 6 locations in west-central North America (Table II; Fig. 1). Based on observations of DSL in feces of wild cervids and mountain goats, a more widespread distribution of *P. odocoilei* was suspected, but not confirmed, by adult parasite recovery and identification (Pybus et al., 1984; Lankester, 2001). Efforts to characterize the distribution of *P. odocoilei* in North America have been hampered by unidentifiable, or mistakenly identified, DSL. For example, DSL from Stone's sheep in northern British Columbia were tentatively identified as *Muellerius* sp. (Seip and Bunnell, 1985) but, based on the findings in the present study, likely were *P. odocoilei*. By integrating comparative morphology of adult parasites and molecular identification of first-stage larvae, we defined more thoroughly the geographic distribution of *P. odocoilei* in wild cervids and caprines from western North America.

Molecular identification of DSL

We obtained and compared sequences for the ITS2 region of ribosomal RNA genes of 9 protostrongylid species validated by comparative morphology of adult parasites, and we used this database to identify unknown DSL as *P. odocoilei*. Identifications were based on large differences between representatives of *Parelaphostrongylus* and other genera, and smaller fixed differences among the 3 species of *Parelaphostrongylus*. Interestingly, consistent with the observations by Junnila (2002), no fixed differences were found in the limited number of sequences available for *Elaphostrongylus rangiferi* and *E. cervi*. In combination with controversy over the taxonomy of *Elaphostrongylus* spp. based on adult parasite morphology (Carreno and Lankester, 1993), this suggests that further molecular characterization at multiple loci is needed to define the relationship of these 2 species.

Taxon-level differences among the ITS2 sequences of the Protostrongylidae were roughly comparable to those reported for other nematode parasites, with raw similarities among genera of 30–60% as compared to 60–80% for gastrointestinal trichostrongyles (Heise et al., 1999). Similarity among species of *Parelaphostrongylus* was 90–98%, which is comparable to the 89–99% reported among gastrointestinal trichostrongyles (Hoste et al., 1995; Newton et al., 1998; Heise et al., 1999). *Elaphostrongylus alces* had only 63–65% similarity with *E. rangiferi* and *E. cervi*, which is closer to the similarity (44–79%) among species of *Dictyocaulus* (Hoglund et al., 2003) and among genera of the Protostrongylidae. Caution must be exercised when comparing sequences outside the Elaphostrongylinae, which were sufficiently divergent from the Elaphostrongylinae to preclude reliable alignment. In addition, to our knowledge, genetic ''yardsticks'' for ITS2 sequence divergence have not yet been developed for nematodes (Anderson et al., 1998; Monis et al., 2002; Hoglund et al., 2003). Finally, genetic distances based on limited sequence data from a single locus cannot be used to infer phylogenetic relationships; decisions about species diversity and validity should be based on multiple loci and interpreted within a sound phylogenetic framework (Nadler, 2002).

Based on ITS2 sequences of a protostrongylid parasite across its known geographic range, we describe levels of intraspecific polymorphism within *P. odocoilei* higher than those that have been previously reported (Junnila, 2002) but comparable to those reported for gastrointestinal nematodes (Heise et al., 1999) and for *Dictyocaulus eckerti* (Hoglund et al., 1999). Intraspecific polymorphism likely accounts for small differences in length of the ITS2 region of *P. odocoilei* within the present study (557–562 bp) (Table III) and between ITS2 sequences of protostrongylids in both the present and previous studies (Junnila, 2002). The lengths reported by Gajadhar et al. (2000) were estimates using the relative positions of bands on a gel (Table III). Intraspecific polymorphism within *P. odocoilei* resulted, in part, from 5 single-nucleotide polymorphisms in cloned ITS2 sequences, which corresponded to overlapping traces when PCR products were sequenced directly. By sequencing clones of ITS2 from an individual adult female nematode, we confirmed the presence of 2 distinct copies of ITS2, which differed at these 5 polymorphic sites, within the individual genome (GenBank AY648401 through AY648406). The ITS2 ''types'' have been reported in other nematode species (Divina et al.,

2000), and such heterogeneity among rDNA copies may persist within a lineage for more than a million generations (Coen et al., 1982). Only those differences that have become fixed between related lineages provide positive evidence for their differentiation (Williams et al., 1988; Rich et al., 1997; Santin-Duran et al., 2002).

We observed no fixed differences in sequence of ITS2 of *P. odocoilei* relative to host or geographic location, despite the broad distribution of this parasite from California to Alaska. The ITS2, although useful for species identification, appears to be unsuitable for detecting population genetic structure (Hoste et al., 1993; Anderson et al., 1998). In addition, the minimal level of genetic diversity of the ITS2 among the elaphostrongylines explains why only sequencing, and not differences in the mobility of PCR products of ITS2 through conventional agarose gels, could distinguish the 3 closely related species of *Parelaphostrongylus* (Gasser and Hoste, 1995; Gajadhar et al., 2000). Sequencing is both time and labor intensive, and mixed infections may not be detected unless large numbers of larvae are analyzed. If PCR product from samples of pooled larvae is sequenced directly instead of cloned, then overlapping electrophoretograms may reveal the presence of mixed infections (Junnila, 2002; unpubl. obs.). PCR in combination with species-specific primers, restriction fragment length polymorphism, or single-strand conformation polymorphism, would significantly increase the ability to identify species of protostrongylids, especially in mixed infections (Gasser and Monti, 1997; Monis et al., 2002). Finally, a mitochondrial locus such as cytochrome oxidase, which is conserved less strongly, is better suited for determining the presence of population genetic structure or cryptic species and for addressing phylogenetic hypotheses (Avise, 1994; Anderson et al., 1998; Hoberg et al., 1999; Blouin, 2002).

Epizootiology of the protostrongylids *P. odocoilei* and *Protostrongylus* spp.

We describe the epizootiology (i.e., prevalence, host associations, and geographic distribution) of *P. odocoilei* identified using molecular analyses of DSL and of *Protostrongylus* spp. identified using larval morphology. Larvae of *Protostrongylus* spp. were recovered from fecal samples from all thinhorn sheep, bighorn sheep, and mountain goat populations examined. Shedding of *Protostrongylus* spp. larvae in feces is affected by season; therefore, despite variability of the data in Table II, close to 100% of thinhorn sheep, like bighorn sheep, likely are infected with *Protostrongylus* spp. (Forrester and Senger, 1964; Uhazy et al., 1973; Pybus and Shave, 1984). The larvae recovered likely were *P. stilesi*, *P. rushi*, or both. These larvae are morphologically indistinguishable, and both species have been reported in these hosts (Uhazy et al., 1973; Samuel et al., 1977; Pybus et al., 1984; Kutz et al., 2001). Using NC1 and NC2 primers, we could not amplify the ITS2 region of larvae of *Protostrongylus* spp. and, therefore, could not differentiate larvae of *P. stilesi* from those of *P. rushi* using molecular analyses.

The DSL were present in 80–100% of samples from Dall's sheep and mule deer, but prevalence was more variable in samples from Stone's sheep (66–100%), mountain goats (41–100%), woodland caribou (28 and 43%), and black-tailed deer (25 and 56%). Logistical constraints generally entailed identification of DSL from only a single host at each location. Therefore, the prevalence of DSL, which were assumed to be *P. odocoilei*, should be interpreted considering the possibility of infection with other protostrongylids, such as *P. andersoni*, which has been reported in woodland caribou elsewhere in Canada (Lankester and Fong, 1989; Lankester and Hauta, 1989) and in experimentally infected mule deer (Pybus and Samuel, 1984a). In addition, although we standardized the methods used for larval recovery as much as possible, sample age and storage were somewhat variable. Season of collection and host factors, such as age and sex, influence larval shedding (Uhazy et al., 1973; Samuel et al., 1985; Festa-Bianchet, 1991; Peterson et al., 1996; Forrester and Lankester, 1997). Despite these sources of variability, the high prevalence of DSL in Dall's sheep and mule deer suggests that they are equally suitable hosts. Lower prevalence in Stone's sheep, mountain goats, black-tailed deer, and woodland caribou in both the present and previous studies suggests that they may be less suitable hosts, either inherently or because of differences in exposure resulting from behavior, habitat use, or host density.

No DSL were recovered from any of the 3 bighorn sheep populations examined in the present study. Indeed, DSL have been observed only rarely in fecal samples from bighorn sheep across North America, which are monitored extensively to determine the prevalence and intensity of *Protostrongylus* spp. larvae. The DSL have been reported, but not identified, in bighorn sheep from East Kootenay and Premier Ridge, British Columbia, Canada; Banff, Alberta, Canada; Lower Rock Creek, Montana; and North Dakota (Hudson et al., 1972; Pybus and Shave, 1984; Aune et al., 1998). Identification based on recovery of adult parasites has been accomplished only in bighorn sheep in South Dakota, which proved to be infected with *Muellerius capillaris* (Pybus and Shave, 1984). Many populations of bighorn sheep likely have been exposed to *P. odocoilei* by sharing range, at least seasonally, with infected mule deer and mountain goats (e.g., sites 20 and 28 in Table II and Fig. 1). Currently, however, no evidence indicates that bighorn sheep, unlike thinhorn sheep and mountain goats, are hosts for *P. odocoilei*. This is surprising in light of the broad host range of *P. odocoilei* and the similarity of the endemic parasite fauna of thinhorn sheep, bighorn sheep, and mountain goats (Samuel et al., 1977; Hoberg et al., 2001). The suitability of bighorn sheep as a host for *P. odocoilei* warrants further investigation.

No DSL were recovered from several populations of thinhorn sheep, despite repeated survey and adequate sample size, with the latter based on high prevalence (80–100%) of DSL in infected thinhorn sheep populations elsewhere (Table II; Fig. 1). In several instances, uninfected populations of thinhorn sheep were in close proximity to infected populations. Parasite transmission may not occur between these populations because mountain sheep exhibit high fidelity to their seasonal ranges (Geist, 1971; Valdez and Krausman, 1999). This philopatry is consistent with a genetic structure suggesting isolation and limited opportunities for gene flow and, by extension, parasite transmission among thinhorn sheep populations in close proximity (Worley et al., 2004). Also, geographic barriers to sheep movement exist between sheep in the Nahanni range, where DSL were not recovered, and those in the Mackenzie Mountains, where *P. odocoilei* is well established (N. Larter, pers.

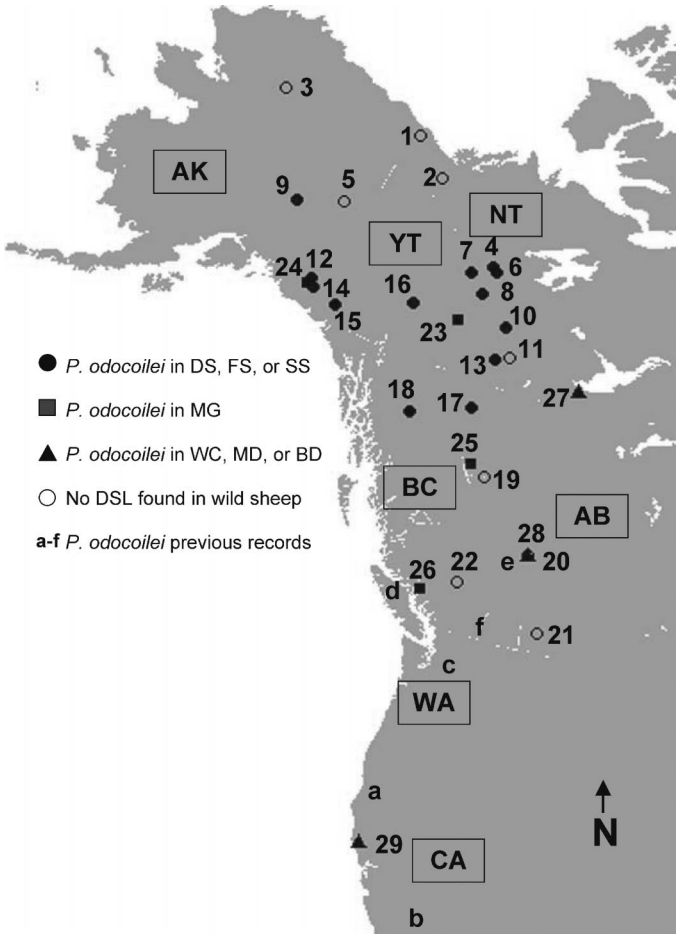

FIGURE 1. Known geographic distribution of *Parelaphostrongylus odocoilei* in North America. For abbreviations, numbers, and letters, see Table II.

comm.). Currently, no geographic barriers are evident between an uninfected population of Stone's sheep at Williston Lake and nearby infected mountain goats and Stone's sheep. Sheep are sparse at the southern end of the range, however, and habitat-sharing may not occur among herds (Shackleton, 1999; J. Elliott, pers. comm.).

Samples from Dall's sheep north of the Arctic Circle (Baird, Brooks, British, and Richardson Mountains) were negative for DSL (Table II; Fig. 1). *Muellerius* sp., which produces DSL, has been reported in Dall's sheep from the Brooks Range, Alaska (Dau, 1981). This record was based on findings at necropsy, however, and lung lesions typical of *Protostrongylus* sp. might have been identified mistakenly as those of *Muellerius* sp. *Parelaphostrongylus odocoilei* may be absent in the geographically isolated populations of Dall's sheep north of the Arctic Circle, either because it has never been introduced or because it could not establish in this high-latitude environment. The latter explanation is less likely, because favorable abiotic conditions and gastropod intermediate hosts suitable for transmission of some species of protostrongylids in the arctic regions may be inferred from the presence of *Protostrongylus* spp. in these populations of Dall's sheep and of other protostrongylids in muskoxen and caribou (Hoberg et al., 1995, 2002; Lankester, 2001).

To explain the historical origins and current distribution of *P. odocoilei* in North America, phylogeographic and population genetic studies of both parasite and hosts are needed (Avise, 2000; Hoberg et al., 2003). Our finding that *P. odocoilei* is well established in western North America is consistent with Platt's (1984) hypothesis that *P. odocoilei* developed with the ancestor of mule deer endemic to this region. Mule deer do not, however, currently share range with Dall's sheep, and mule deer have a limited range overlap with Stone's sheep (Shackleton, 1999; Kutz et al., 2001). The widespread distribution of *P. odocoilei* in thinhorn sheep cannot be explained by recent transmission of *P. odocoilei* from mule deer. Interestingly, Platt (1984) suggests that first-stage larvae of *P. odocoilei* are freeze-tolerant relative to those of *P. tenuis* (Shostak and Samuel, 1984), and this may account for the successful establishment of *P. odocoilei*, but not of *P. tenuis*, in northern North America.

Application of morphological and molecular techniques in parasitology

Molecular identification, validated by adult parasite morphology, of unknown protostrongylid larvae in a broad-based survey illustrates the concept of ''epizootiological probes,'' which have many applications in parasitological studies (Divina et al., 2000; Hoberg et al., 2001, 2003). The literature includes many reports of unidentifiable first-stage larvae of protostrongylid parasites in North American wildlife (Lankester et al., 1976; Pybus and Samuel, 1981; Pybus et al., 1984; Gray et al., 1986). In addition to first-stage larvae, third-stage larvae from gastropod intermediate hosts, adult female nematodes, and partial nematodes now can be identified. Applications in wildlife management and conservation biology include diagnosis of parasites in hosts that cannot be sacrificed for adult parasite recovery, such as endangered species or those that inhabit remote locations, and in risk assessments before translocation of either wild or game-ranched animals (Hoberg et al., 2001, 2003). The use of noninvasive molecular techniques for identifying protostrongylid parasites represents a breakthrough for this group of nematodes, which hitherto has been diagnostically intractable. Further refinement of molecular techniques to allow detection of mixed infections and to minimize both cost and time (i.e., for sequencing) are needed before they can be put to widespread use for identifying parasites of wild, game-ranched, and domestic animals, particularly in areas of natural range overlap and at the interface of managed and wild systems.

In addition to these practical diagnostic applications, epizootiological probes finally will allow comprehensive descriptions of the host associations and geographic distributions of parasites. The present study has greatly increased the known geographic distribution of *P. odocoilei* in North America, but the sequence of first-stage larvae from mule deer in Montana matching that of *P. odocoilei* (Junnila, 2002) suggests that the full range of this parasite has yet to be described. As molecular identification of first-stage larvae becomes more feasible and more widely applied, *P. odocoilei* and other protostrongylids likely will prove to have broader and more overlapping distributions than was previously suspected. For example, DSL from barren-ground caribou in northwestern Canada (Yukon Territory and Northwest Territories) were assumed to be *P. andersoni*, which has been reported in the Beverly herd in central

Northwest Territories and, based on unpublished reports, in caribou in Alaska (Lankester and Hauta, 1989; Lankester, 2001). Recent molecular characterization of these DSL not only has confirmed the presence of *P. andersoni* but has led to the discovery of a previously undescribed species of protostrongylid in barren-ground and woodland caribou at multiple locations in northwestern Canada (unpubl. obs.).

Knowledge of the species of parasite(s) in a region is necessary to identify risks of parasite transmission and disease emergence under changing environmental conditions (Hoberg, 1997; Hoberg et al., 2003; Kutz, Hoberg et al., 2004). Several protostrongylids are linked to neurological disease syndromes, and all have the potential to cause pulmonary pathology (Lankester, 2001). *Parelaphostrongylus odocoilei*, for example, can cause respiratory failure in both experimentally and naturally infected hosts (Brunetti, 1969; Platt and Samuel, 1978b; Pybus et al., 1984; Pybus and Samuel, 1984b). Stress from environmental factors, in combination with various bacteria and protostrongylid lungworms, has been linked to pneumonia epizootics in bighorn sheep, and isolated cases of fatal pneumonia in wild Dall's sheep have been reported (Bunch et al., 1999; Jenkins et al., 2000). Outbreaks of clinical cerebrospinal elaphostrongylosis in reindeer in Norway (Handeland and Slettbakk, 1994), as well as accelerated development (from a 2- to a 1-yr cycle) of the muskox lungworm *Umingmakstrongylus pallikuukensis* in Nunavut, Canada (Kutz, Hoberg et al., 2004), have been linked to warmer summer temperatures. In addition to unprecedented rates of warming, northern ecosystems are facing disturbance from increasing oil, gas, and mineral exploration.

Shifts in host distributions, possibly in response to habitat disruption such as that described above, and the breakdown of isolation could lead to exchange of parasites such as *P. odocoilei* and even disease outbreaks (Rausch, 1972; Hoberg, 1997; Hoberg et al., 2002). For example, muskox populations are expanding across the Arctic, and soon, both subspecies of muskoxen in the Northwest Territories (*Ovibos moschatus wardi* and *O. m. moschatus*) may bridge the gap between thinhorn sheep populations in the Mackenzie Mountains infected with *P. odocoilei* and naïve populations in the Richardson Mountains. Although it is not known if muskoxen are suitable hosts for *P. odocoilei*, muskoxen and Dall's sheep share some species of gastrointestinal parasites and the protostrongylid lungworm *Protostrongylus stilesi* (Hoberg et al., 2001, 2002; Kutz, Garde et al., 2004).

Epizootiological investigations employing techniques from traditional parasitology combined with modern molecular analyses, such as the present study, are needed to characterize the parasite fauna of wildlife and their geographic distributions. Such knowledge is key to understanding how host–parasite assemblages originated and to predicting how they may respond to environmental change, an increasingly recognized concern for wildlife management and conservation.

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